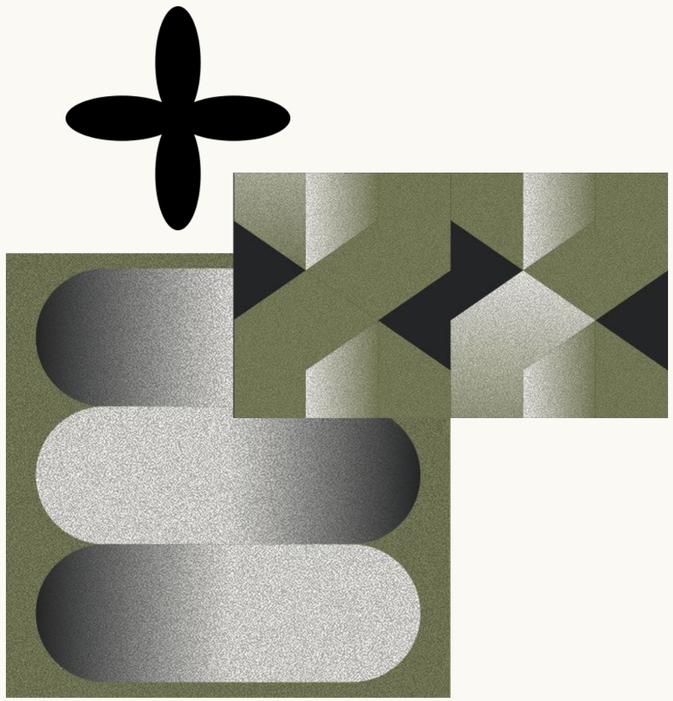
miRNA therapy for BACTERIAL PRODUCTION AND DELIVERY

iGEM Club, IITD

2024

Colorectal Cancer(CRC)



01

Prevalence

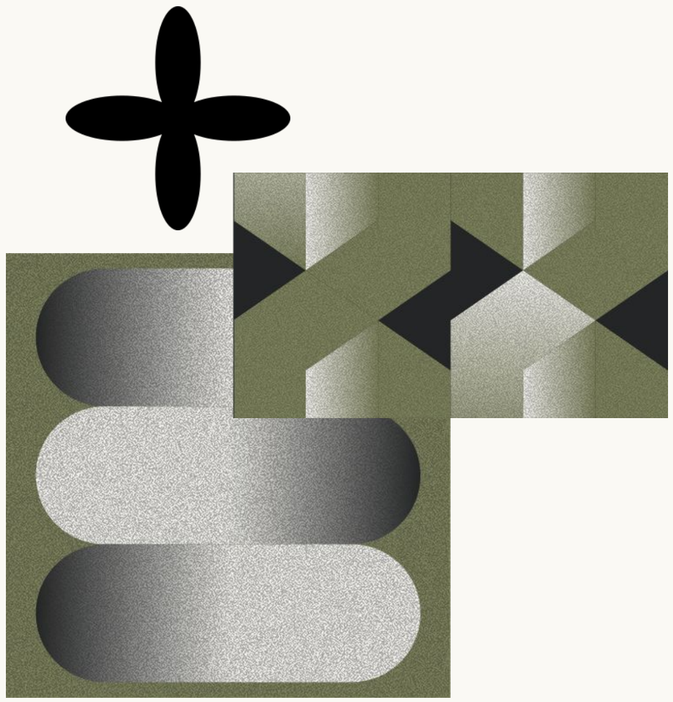
CRC is the third most commonly diagnosed cancer worldwide, with over 1.9 million new cases and nearly 935,000 deaths reported in 2020. According to the Indian Council of Medical Research (ICMR), the incidence of CRC in India is projected to increase by 128% in men and 125% in women by the year 2030.

02

Challenges in diagnostics

The National Cancer Registry Program of India reported that nearly 57% of CRC cases in India are diagnosed at an advanced stage, leading to poorer outcomes. Limited awareness, inadequate screening programs, and uneven distribution of healthcare facilities are reasons behind this low diagnosis rate.

Existing Therapies



01

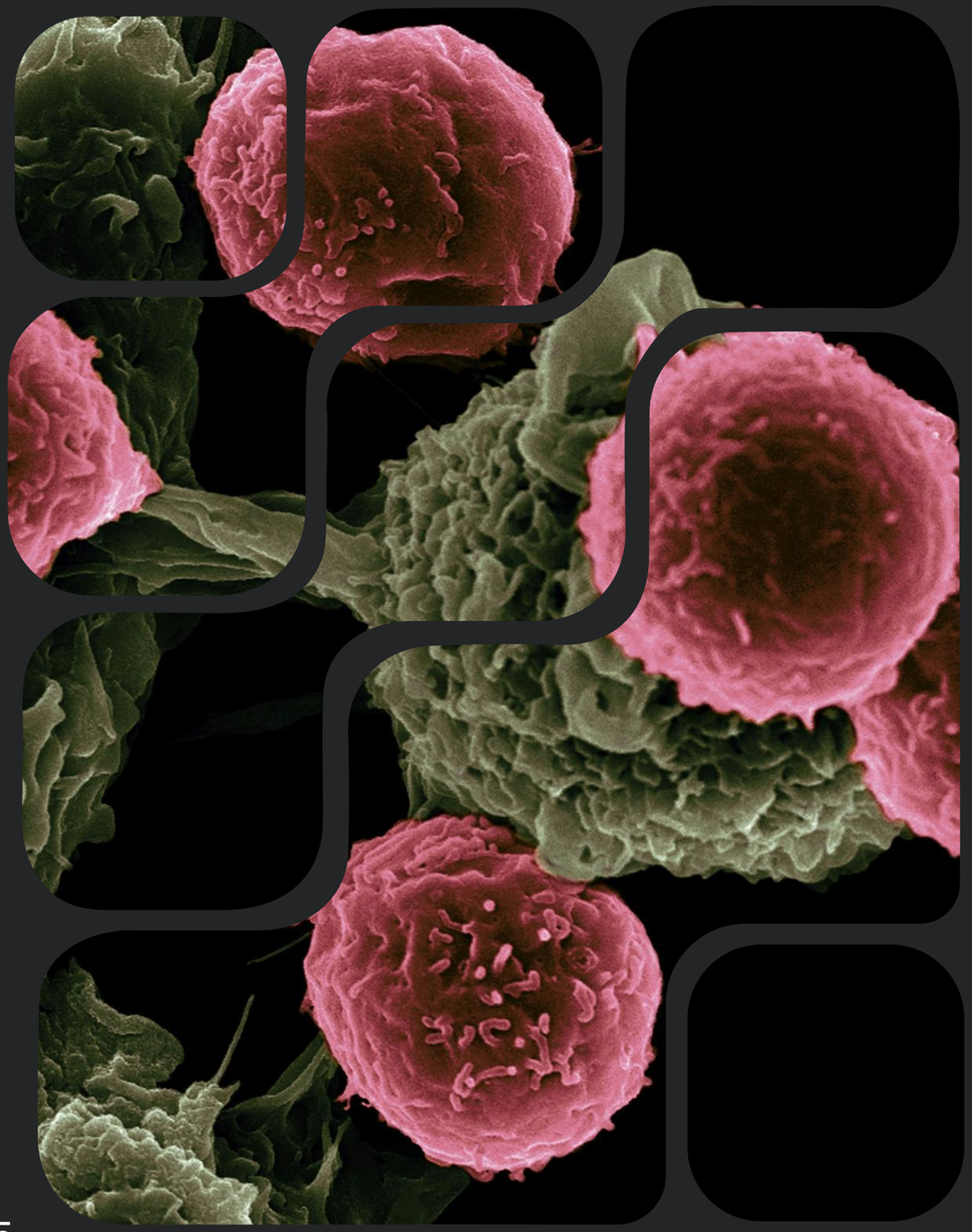
Major modalities of treatment

Existing therapies for CRC include surgery, chemotherapy, radiation therapy, targeted therapy, and immunotherapy. While these treatments have shown effectiveness in managing CRC, they also have various disadvantages.

02

Disadvantages of major modalities

- Surgery, the primary treatment for localized CRC, can lead to complications such as infections, bleeding, and damage to surrounding organs.
- Chemotherapy, often used in combination with surgery, can cause side effects like nausea, hair loss, and fatigue.
- Radiation therapy, while effective in treating specific cases of CRC, can damage healthy tissues surrounding the tumour site.
- Targeted therapy and immunotherapy have shown promising results in some CRC patients, but not all patients respond to these treatments, and they can be costly and have long-term side effects.

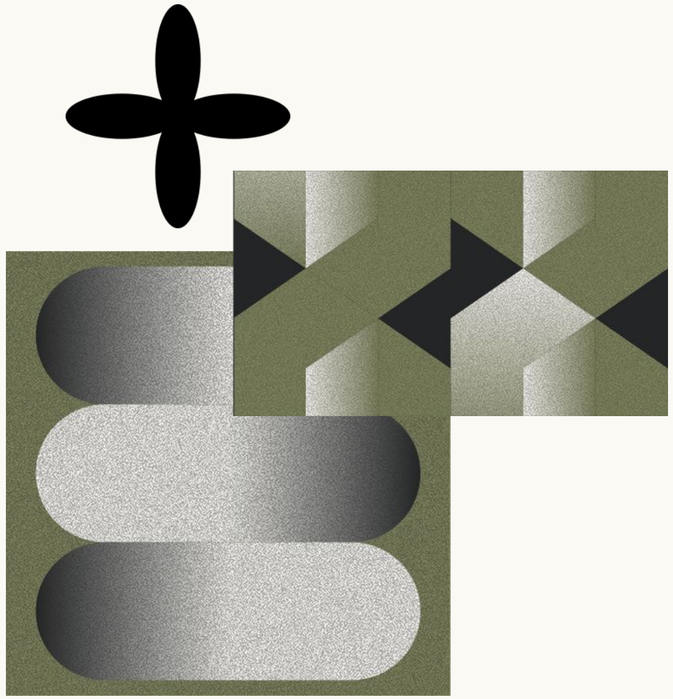


Why Gene Therapy?

FUTURE OF CANCER TREATMENT

Gene therapy holds great promise for revolutionizing the treatment of CRC by addressing the limitations of existing therapies and offering targeted and personalized treatment strategies.

Gene Therapy



01 Advantage of gene therapy

One of the significant advantages of gene therapy is its potential for targeted treatment, where therapeutic genes are delivered directly to cancer cells, minimizing damage to healthy tissues. Gene therapy can also be tailored to individual patients based on their genetic makeup and tumor characteristics, leading to personalized treatment strategies.

02 Gene Therapy modalities

Some existing delivery methods include viral vectors, non-viral vectors, and RNA-based therapies.

- Viral vectors face the issue of limited capacity for large genes and they might trigger immune responses.
- Non-viral vectors, like liposomes and nanoparticles, offer the advantage of reduced immunogenicity and toxicity, but they face issues in transfection efficiency and stability.

03 Using RNAi for gene therapy

RNA-based therapies, such as small interfering RNAs (siRNAs) and messenger RNAs (mRNAs), have emerged as promising tools for gene therapy in CRC.

Our Solution

E. coli Nissle 1917 as Producer and Carrier of miRNA to the Cancer Cells

Part 1
Project Description

Why E. coli Nissle? What is OMV?

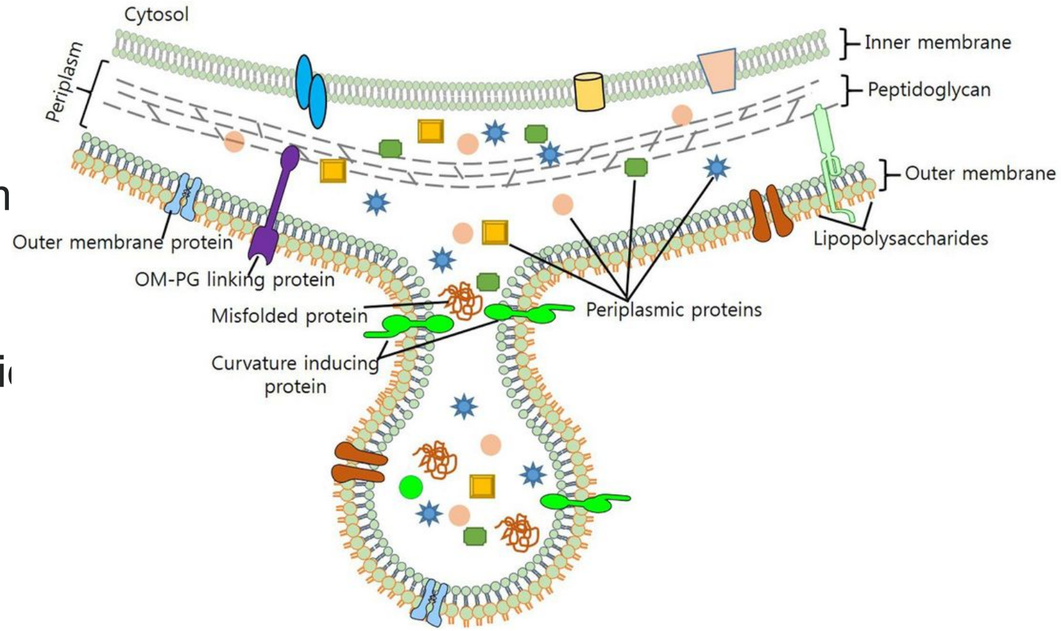
EcN is a Probiotic Bacteria, already a part of Gut Microbiome

It is a GRAS (Generally Regarded As Safe) Strain of E. coli.

OMVs or Outer Membrane Vesicles are nanoscale spherical vesicles released from Gram-negative bacteria.

These can be engineered to display specifi targeting Affibodies.

It is prevalently used as a mode of drug delivery



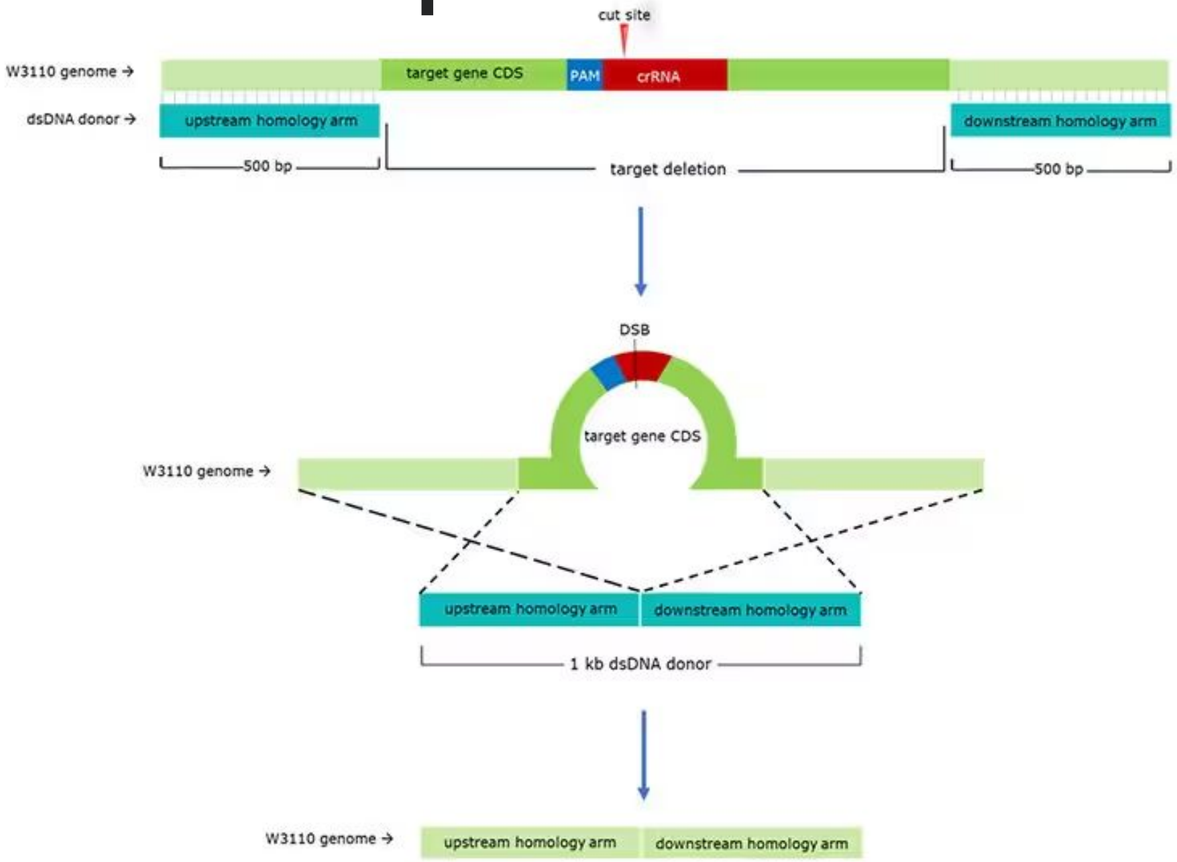
Development of Novel Chassis for in vivo OMV overproduction

Many Bacteria natively produce OMVs (which includes EcN). But for drug delivery, we require a larger number of it.

tolA gene K/O in E. coli have been shown to increase the OMV production in E. coli (especially K-12)

For this we need to construct the sgRNA containing plasmid and the Cas9 Plasmid.

Substituting the gene with lambda-Red is quite prevalent as a



tolA K/O

miRNA

Recombinant RBP

Surface Nanobodies

Cre-Lox Gene Regulation

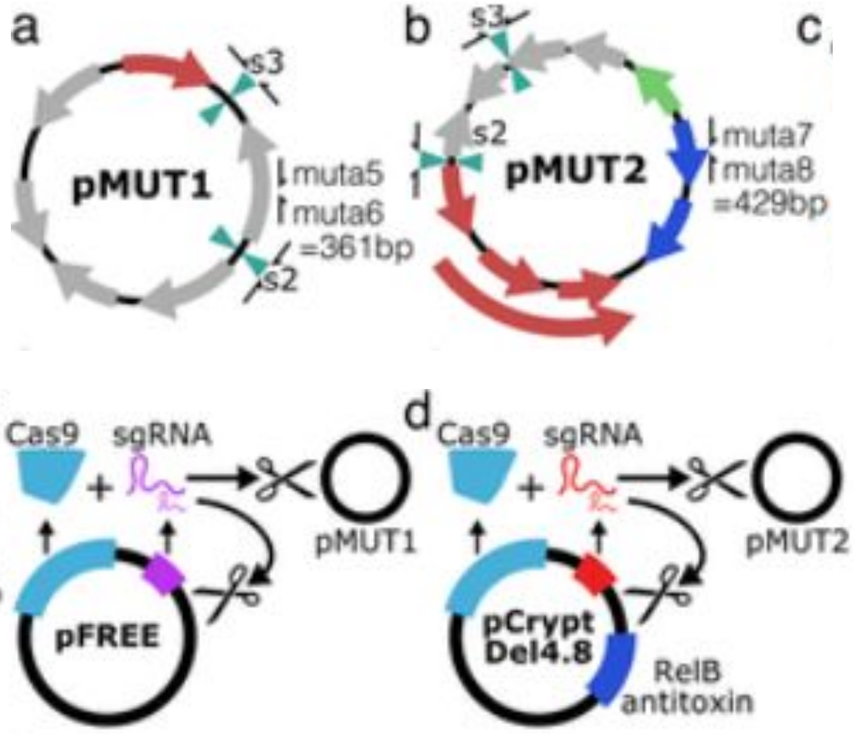
Development of Novel Chassis for in vivo OMV overproduction

Also, EcN has pMUT1 and pMUT2 plasmids constitutively.

So, before proceeding we would need to cure the EcN of plasmids.

CRISPR-Cas9 based Curing:-
2 Plasmids Required

1. pFREE
2. pCryptDel4.8



miRNA for Therapy of CRC

There are many miRs in CRC cells which are over and under expressed. As our model would be most suitable for delivery of miRs to the Cancer cells, we would be targeting the down-regulated miRs, specifically miR-145.

Length of miR-145 gene :- 1 . 8 kB

And also a short RBP homing sequence needs to be added (Length can be decided after further discussion)

Table 3 Expresssion of microRNAs in colorectal tumors

	Colorectal cancer (n = 63)	*Colorectal adenoma (n = 65)*	*P-value*
miR-143↓	45/63 (71.4%)	43/65 (66.2%)	0.5198
miR-145↓	49/63 (77.8%)	45/65 (69.2%)	0.2737
miR-7↑	46/63 (73.0%)	19/65 (29.2%)	<0.0001
miR-21↑	20/63 (31.7%)	33/65 (50.8%)	0.0289
miR-34a↓	41/63 (65.0%)	21/65 (32.3%)	0.0002
miR-143↓+ miR-145↓	45/63 (71.4%)	42/65 (64.6%)	0.4089
miR-7↑+ miR-34a↓	29/63 (46.0%)	2/65 (3.1%)	<0.0001
miR-143↓+ miR-145↓ + miR-7↑	31/63 (49.2%)	5/65 (7.7%)	<0.0001
miR-143↓+ miR-145↓+ miR-21↑	9/63 (14.3%)	14/65 (21.5%)	0.2853
miR-143↓+ miR-145↓ + miR-34a↓	33/63 (52.3%)	20/65 (30.8%)	0.1308
miR-143↓+ miR-145↓+ miR-7↑ + miR-34a↓	21/63 (33.3%)	1/65 (1.5%)	<0.0001
miR-143↓+ miR-145↓+ miR-7↑ + miR-34a↓ + miR-21↑	2/63 (3.2%)	1/65 (1.5%)	0.5407

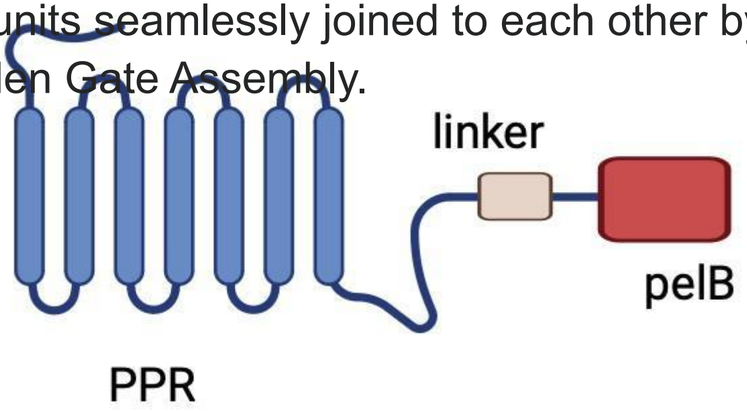
Recombinant RBP

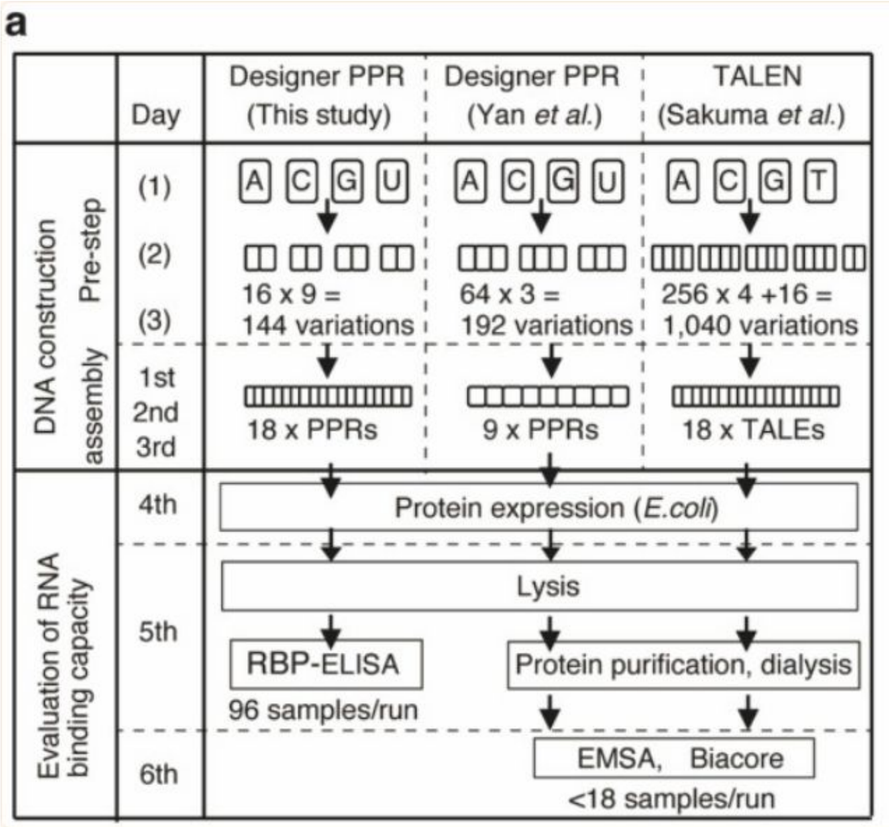
Multiple RNA Binding Proteins are present in E. coli. PPR is one of them. (It is a homo-multimer).

PPR is highly versatile for Engineering Binding Specificity to a particular RNA sequence.

Each subunit can be engineered to be specific to particular Nucleotide of RNA.

Subunits seamlessly joined to each other by Golden Gate Assembly.





Recombinant RBP

Here is a brief description on how we are planning on achieving the assembly.

At the end of RBD, a pelB sequence also needs to be added.

PelB is a signalling peptide
Carries attached proteins to the Periplasmic Space

Length of PelB :- 22 Residues

PelB :- MKYLLPTAAAGLLLLAAQPAMA

Length of one subunit of PPR :- 35 Residues

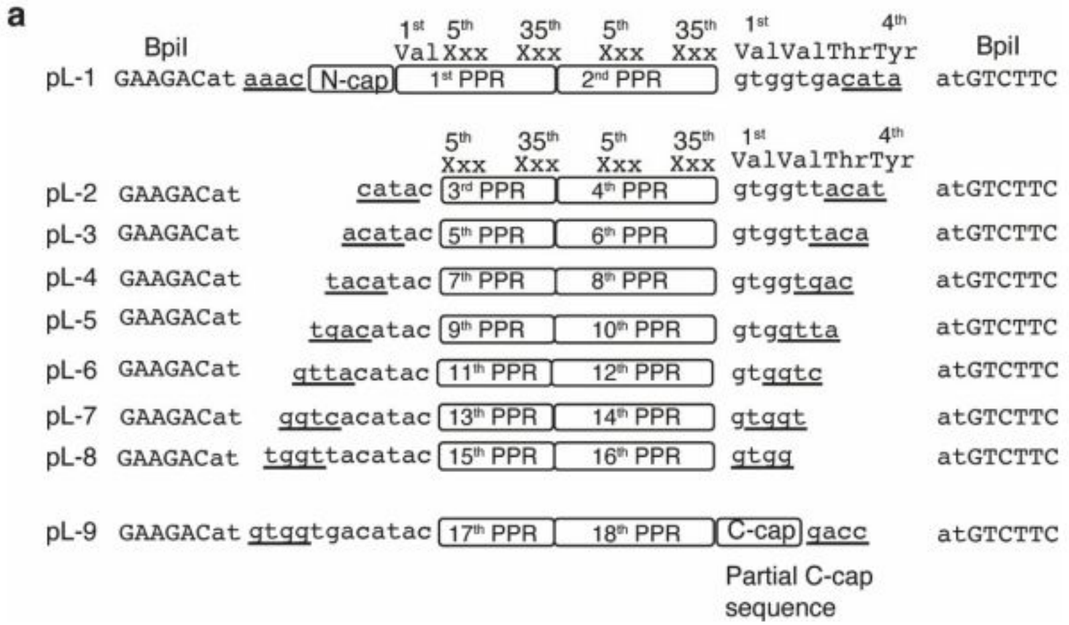
PPR2.0_A (for adenine):

VVTYTTLIDGLCKAGDVDEALELFKEMRSKGVKPN

PPR2.0_C (for cytosine): VVTYNTLIDGLCKSGKIEEALKLFKEMEEKGITPS

PPR2.0_G (for guanine): VVTYTTLIDGLCKAGKVDEALELFDEMKERGIKPD

PPR2.0_U (for uracil): VVTYNTLIDGLCKAGRLDEAFELLEEMEEKGIKPD



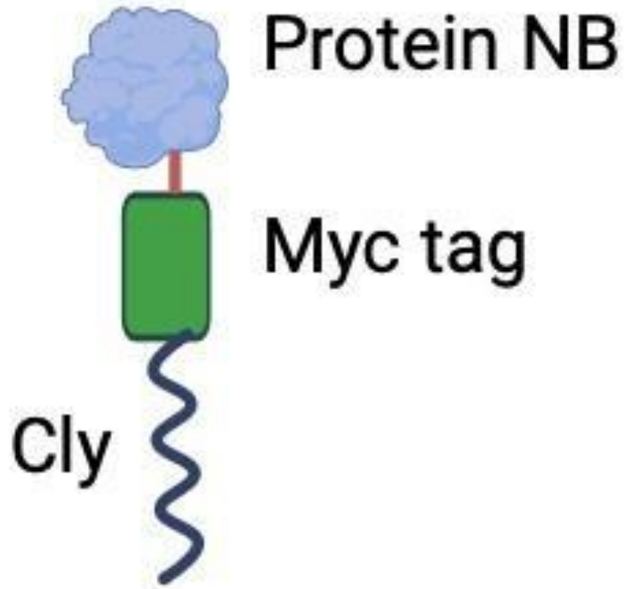
Surface Expression of Affinity Nanobodies

For increasing the specificity of OMV-based therapy, we need to add some surface receptors for target Cell Surface Antigens.

Overexpression of EGFR is prominent for CRC cells.

Nanobodies have been created having high binding affinity towards EGFRs. Particularly, VEGFR1 is shown to have a high efficacy.

Fusion Protein needs to be created of Nb with Cly-Protein (a surface Protein of E. coli) and a Myc Tag for easier Western blot.

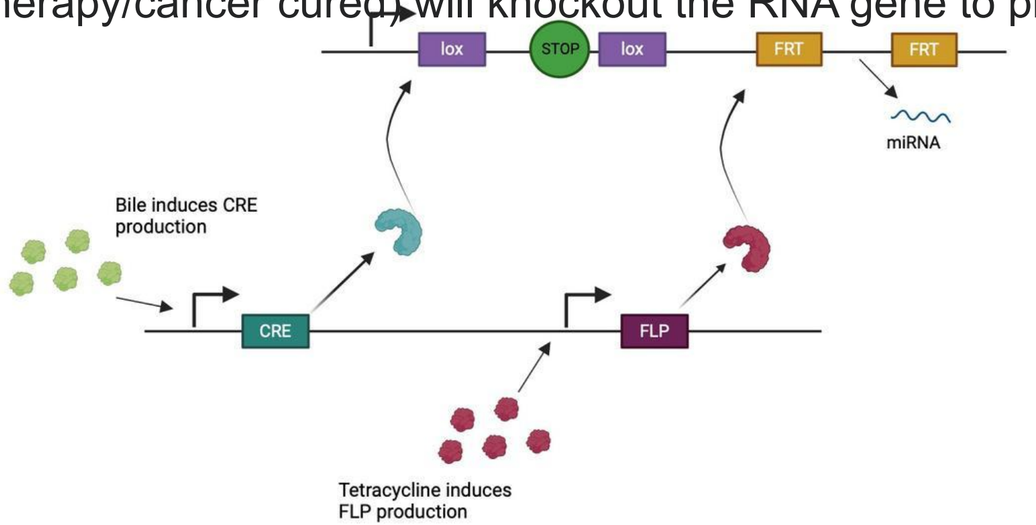


Nb name	Amino acid sequence of CDR-H3	Number of clones	
		MACS	CellS
VEGFR1	DKWSSSRRSVDYDY	72/96	18/96
VEGFR2	TYNPYSRDHYFPRMTTEYDY	10/96	03/96
VEGFR3	RYSDVIFTLPERYAY	01/96	—
VEGFR4	STYSRDSVFTKWANYNY	03/96	02/96
VEGFR5	GPILGSSESYRSSRRYAY	01/96	—
VEGFR6	DLNFIGIVTTTSEKYDY	—	02/96

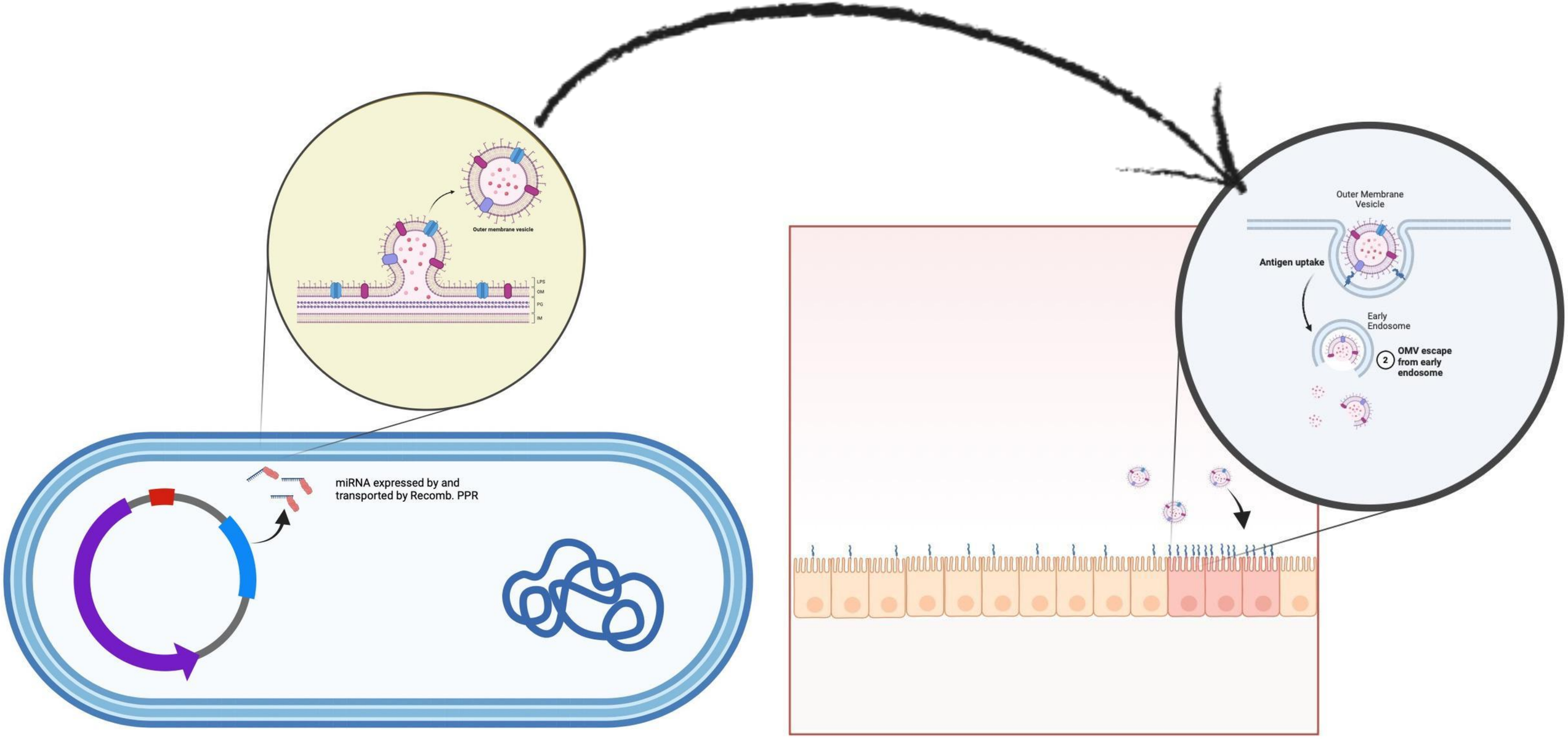
Recombinase switches for regulation via in-vivo knockout

One time knockouts of two separate sequences
One to kickstart miRNA production and other to irreversibly stop it

Bile inside gut will start constitutive expression (when the bacteria is first planted in human gut) by excising stop signal
Tet (at the end of therapy/cancer cured) will knockout the RNA gene to prevent metabolic burden .



Cre-Lox Gene Regulation


Outer membrane vesicle
LPS
OM
PG
IM
miRNA expressed by and
transported by Recomb. PPR
Outer Membrane
Vesicle
Antigen uptake
Early
Endosome
2
OMV escape
from early
endosome

Expression Vectors

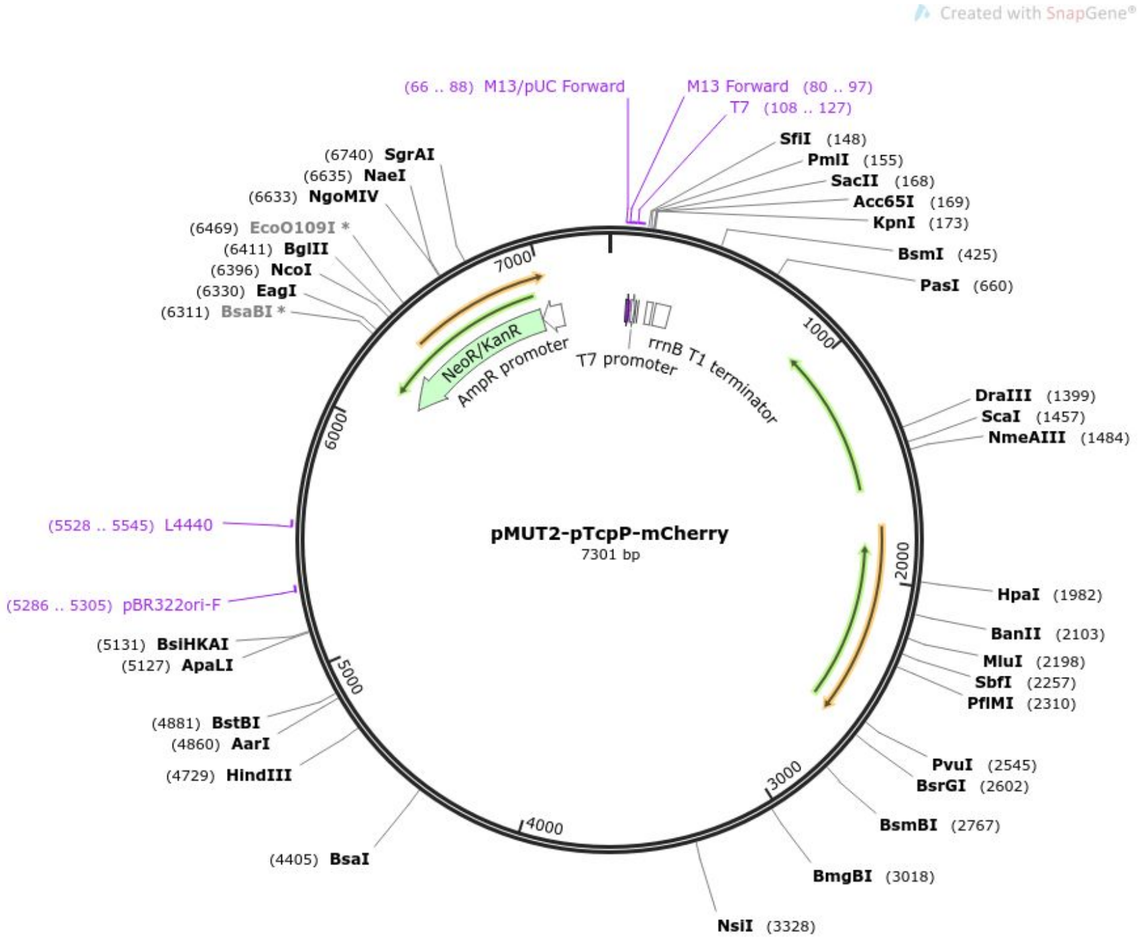
Expression Vectors :- pMUT1 and pMUT2
Because already present in EcN and is also a Cryptic Plasmid

Cryptic Plasmids are Antibiotic-Resistance-Free Plasmids, usually used for Probiotic purposes.

Also EcN retains pMUT1 and pMUT2 upto 90 generations without any external pressure

Cloning in pMUT2-pTcpP-mCherry :-

It has a bile inducible promoter, which we can use to induce the production of Cre Protein.



Expression Vectors

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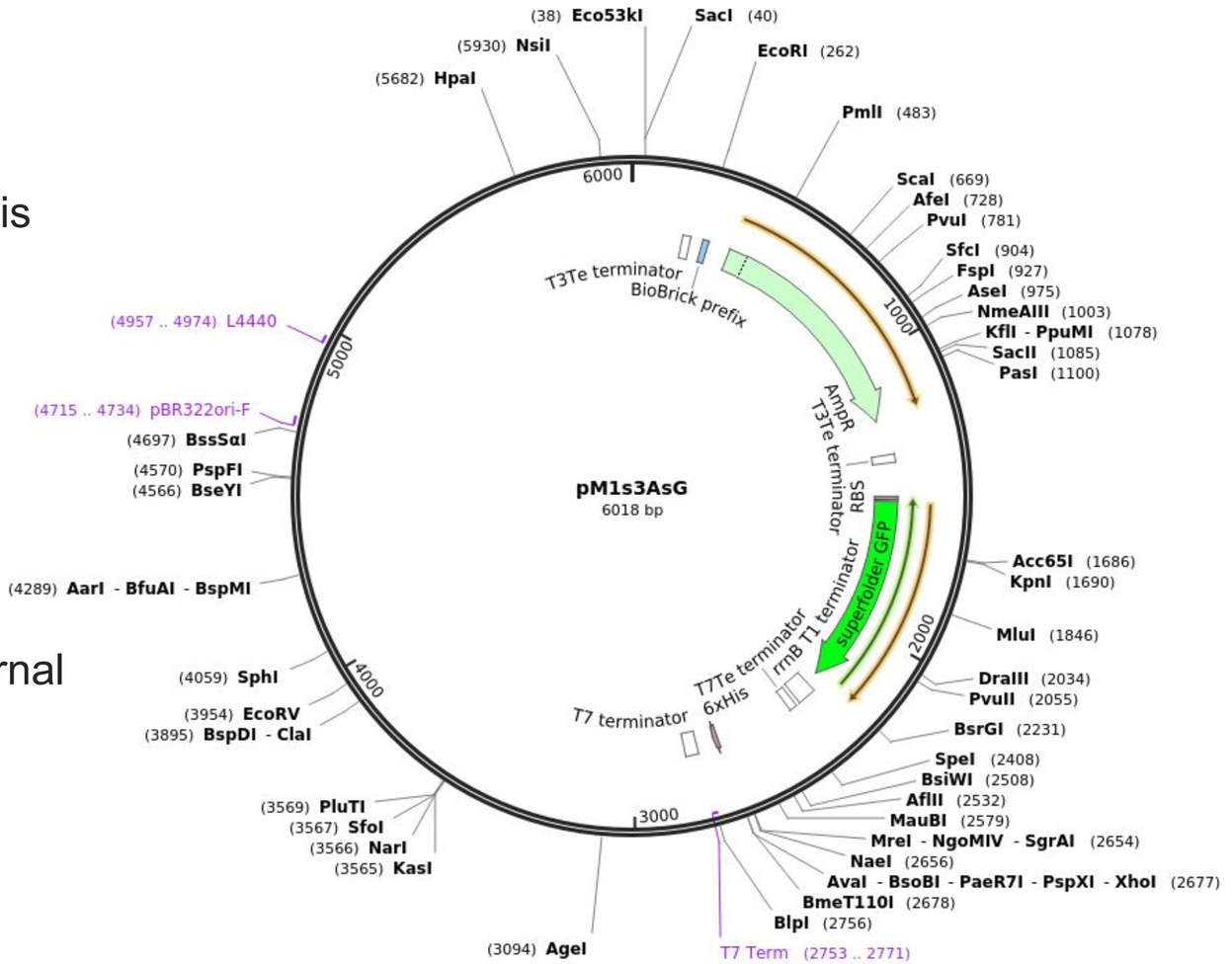
Cloning of pM1s3AsG :-
2 clones :-

1. Recombinant PPR
2. Surface Nanobodies



OBJECTIVE	DESCRIPTION	TEST FOR POC	TIMELINE
Development of Novel Chassis for in vivo OMV Production (in EcN)	Knockout of tolA gene for OMV overproduction; Curing of pMUT1 and pMUT from EcN.	1. lambda-Red based Detection of K/O 2. SDS-PAGE and Western Blot for OMV Overproduction Detection 3. FISH for detection of oriC of Plasmids.	K/O:- Mid-April to Majors; Majors to Late-May Curing:- Late-May to Late June
Recombinant RBP (in K-12)	PPR protein design for specific RNA sequence.	1. Flouroscent tag detection 2. EMSA for detecting the binding of Protein to RNA	Simulation:- Early April to Majors Cloning and Testing:- Late May to late June
Surface Expression of Affinity Nanobodies (in K-12)	Fusing EGFR specific Protein Nanobody with Cly-Protein	Western Blot of Myc Tag present in the fusion protein	Fusion Protein Gene Making:- Early April to Major Cloning and Testing :- Late June to mid July
Recombinase switches for regulation via in-vivo knockout (in K-12)	Cre-Lox based System for Inducible Constitutive Production of miR 145	Checking the miRNA production when induced by Bile.	Design of Complex :- Late May Cloning and Testing :- June to late July
Computational Methods to be implemented	PPR-RNA binding Molecular Dynamics Kinetics modelling of Molecule Production Microbiome Interaction Dynamics Simulation Dosage Simulation	-	Early April to Late July

Part 2
Timelines and Funds for the Project Progress

CONSUMABLES	SOURCE	COST
Plasmids(pFREE, pCryptDel4.8, pMUT2pTcpP-mCherry, pM1s3AsG)	Addgene: pFREE: https://www.addgene.org/92050/ pCryptDel4.8: https://www.addgene.org/141293/ pMUT2pTcpP-mCherry: https://www.addgene.org/192861/ pM1s3AsG: https://www.addgene.org/137921/	1000 USD
Lab Consumables(Enzymes, Petri dishes, Broths, primers, etc.)		4500 USD
Antibodies for OMVs, Anti-Myc and POC Experiments	Sigma Aldrich	2000 USD
Synthesized Genes	IDT (sponsor of iGEM)	

iGEM TIMELINE

RESPONSIBILITY	TIMELINE	STUDENTS RESPONSIBLE	REQUIREMENTS
EC MEETING	MARCH 22	PRATHAM, TUHIN, AJAI	-
TEAM REGISTRATION	APRIL 05	SHASHANK, PRATHAM, VIDYA	6000 USD
IMPACT GRANT	MAY-JUNE	SHASHANK, VIDYA	-
JAMBOREE REGISTRATION	JULY-AUGUST END	SHASHANK, PRATHAM	3000 USD
SAFETY FORM	PRELIMS-5 JUNE FINAL-4 SEP	KUSHAGRA	-
PROMOTIONAL VIDEO	4 SEP	PRATHAM	-
TEAM ROOSTER	11 SEP	TUHIN	-
WIKI	2 OCT	PARTH, KUSHAGRA, RAVINDER	-

Vertical Distribution

VERTICAL	STUDENTS RESPONSIBLE
WET LAB	AMOGH, TUHIN, PRATHAM
DRY LAB	PARTH, KUSHAGRA
HP/EDUCATION	VIDYA, PRATHAM, PARTH
DESIGN/WIKI/WEBSITE	RAVINDER, KUSHAGRA
FUNDRAISING	SHASHANK, PRATHAM, VIDYA

Thank you

Instructors

Prof. Ritu Kulshrestha

Prof. Vivekanandan Perumal

Students

Amogh Varshney

Tuhin Kumar Raut

Prathamdeep Dhanoa

Shashank Choudhary

Parth Patel

Kushagra Patel

Ravinder Kuhad

Vidyadarshini R.